

The flow-cytometric determination of basophil activation induced by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) is useful for *in vitro* diagnosis of the NSAID hypersensitivity syndrome

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Summary

Background Hypersensitivity reactions to non-steroidal anti-inflammatory drugs (NSAIDs), manifested by cutaneous symptoms and/or airway manifestations represent 20–25% of all hypersensitivity reactions to drugs. Today, it is still claimed that no *in vitro* diagnostic tests exist for that condition and that the only way to confirm the diagnosis is a provocation challenge.

Objective The objective of this study was to assess whether NSAIDs may provoke blood basophil activation *in vitro* in such patients, as detected by a flowcytometric technique.

Methods Sixty NSAID hypersensitive patients (38 with cutaneous, 20 with airway and two with cutaneous and airway symptoms) and 30 control patients (15 asthmatics) were selected. Their hypersensitivity was confirmed by documented history indicating at least two clinical episodes to two or more different NSAIDs or by positive oral provocation challenge. Isolated buffy coat leukocytes were stimulated *in vitro* with aspirin, paracetamol, metamizol, diclofenac, and naproxen. The percentage of activated basophils was evaluated by an anti-CD63.

Results Aspirin showed a sensitivity of 43.3%, a specificity of 100%, a positive predictive value of 100% and a negative predictive value of 99.4%. For the other NSAIDs, the sensitivity and specificity values were: for paracetamol 11.7% and 100%, for metamizol 15% and 100%, for diclofenac 43.3% and 93.3% and for naproxen 54.8% and 74.1%. When considering the first four NSAIDs, the global sensitivity raised to 63.3% and specificity to 93.3%. If the number of tests is to be limited for practical reasons, the combination of acetylsalicylic acid and diclofenac at two concentrations yields a sensitivity of 58.3% and a specificity of 93.3%.

Conclusions Flowcytometric determinations of basophil activation following stimulation with NSAIDs show a high sensitivity (60–70%) with specificity above 90%. So this test may help avoiding some cumbersome and dangerous provocation challenges.

Keywords aspirin, basophil activation, CD63, *in vitro* diagnosis, NSAID hypersensitivity

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Introduction

Hypersensitivity to aspirin and other non-steroidal inflammatory drugs (NSAIDs) is a well-known condition, first described as a syndrome by Widal et al. [1] in 1922 and popularized by Samter and Beers [2] in the late 1960s. This syndrome presents either in the airways, as recurrent rhinitis associated with nasal polyposis and followed by asthma attacks induced by aspirin acetylsalicylic acid (ASA) or other NSAIDs (the classical ASA triad) or by skin manifestations such as urticaria and angioedema. Both clinical types may coexist, but this is more seldom. The prevalence of aspirin and NSAID hypersensitivity has been shown in various studies to be about 10–20% of adult asthmatics and 0.6–2.5%

in the general population [3, 4]. It has been reported that untoward reactions to NSAIDs constitute 20–25% of all hypersensitivity responses reported to drugs [5]. The main features, clinical manifestations, pathogenesis, diagnosis, treatment, and prevention of the NSAID hypersensitivity syndrome have been repeatedly reviewed [3, 6].

Hypersensitivity reactions to NSAIDs may occur in an isolated manner, specific for a single drug, or as a syndrome of hypersensitivity to multiple NSAIDs belonging to different pharmacological families [3, 7]. Specific reactions to a single NSAID are likely to be associated with IgE, as has been shown with pyrazolones [8]. With the possible exception of some rare cases of anaphylactic shock to aspirin, which may be associated with specific IgE [9, 10], it is commonly accepted that the syndrome of multiple hypersensitivities to ASA and NSAIDs is not associated with IgE but rests on a different mechanism [3]. It was proposed many years ago [11–13] that the reaction results from inhibition of cyclooxygenase (COX) by ASA-like drugs in the airways or in the skin of

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hypersensitive patients. This basic COX theory has been restricted recently to inhibition of the COX-1 enzyme, which diminishes the production of prostaglandin E₂ (PGE₂) normally acting as a 'brake' on the production of sulphido-leukotrienes (sLT) LTC₄, LTD₄ and LTE₄ and on the release of other mediators by mast cells [3, 14, 15]. In addition, there is evidence that patients hypersensitive to COX-1 inhibitors have a trend to produce higher levels of sLTs even before exposure to ASA/NSAIDs [3, 16]. The fact that sLTs are major mediators of clinical symptoms in hypersensitivity reactions to ASA/NSAIDs is generally recognized [2, 16, 17].

The statement that there is no *in vitro* diagnostic test for that condition and that diagnosis can be established only by provocation challenges with aspirin or NSAIDs has been repeated so often for 20 years, particularly by the well recognized leading authors in the field, Szczeklik in Europe [3, 6] and Stevenson in the United States [18] that it has almost become a dogma for most allergologists. Indeed, classical allergy cellular tests, like histamine release, have yielded mostly negative results in ASA/NSAID hypersensitivity [19, 20]. Several rather anecdotal reports [21–23] and also a few well-controlled and validated studies [24, 25] have clearly shown that ASA/NSAIDs may induce *in vitro*, at least in a sizeable number of ASA/NSAID hypersensitive patients, basophil activation and sLT release. Other authors, however, have apparently not confirmed the phenomenon [26]. Such discrepancies seem to be due primarily to technical differences and different interpretation of the results [27].

In this paper, which encompasses the largest series of *in vitro* diagnostic tests ever reported in patients with clinically validated multiple ASA/NSAID hypersensitivity compared with various categories of controls tolerating ASA/NSAIDs, we have investigated the diagnostic reliability of ASA/NSAID-induced basophil activation determined by flow cytometry.

Material and methods

Patients

The study was performed in 60 patients with established clinical diagnosis of ASA/NSAID hypersensitivity, 27 men and 33 women, with a mean age of 44.1 ± 14.3 years. Thirty-eight patients presented cutaneous symptoms (urticaria–angioedema), 20 respiratory symptoms (bronchial asthma) and two cutaneous and respiratory symptoms. Thirteen of them were atopic.

All the patients stated having experienced urticaria–angioedema or dyspnoea within the first 4 h after NSAID administration. The episodes had been acute in all cases and, except in patient number 7, who required in-hospital assistance because of severe asthma, yielded in less than 24 h with symptomatic treatment. When the symptoms occurred at two or more occasions after administration of at least two NSAIDs belonging to different pharmacological families, the diagnosis of hypersensitivity syndrome to NSAIDs was considered as established, the more as an important proportion of them developed reactions with three or more different NSAIDs. Provocation was not done in these patients because the diagnosis was considered as clinically firmly established. Patients numbers 24, 32, 37 and

60 (Table 1) were nevertheless challenged with ASA in spite of having suffered several adverse reactions with different NSAIDs.

When the symptoms had been presented as a unique episode or more than once but only with a single family of NSAID, controlled oral provocation test was performed with one NSAID belonging to pharmacological family other than the culprit drug. One patient required emergency hospital treatment for severe asthma after NSAID administration, and in this case because of ethical reasons provocation test was not performed (Table 1, case 7).

Confirmatory provocation challenge was performed almost in all the cases with progressive doses of 50, 100, 250 mg ASA or ibuprofen, administered at hourly intervals. If these doses were tolerated, 500 mg were administered after 24 h. Some of our patients presented a positive provocation test to paracetamol or nimesulide, when we investigated their tolerance to alternative NSAIDs. In the rest of the patients, tolerance to paracetamol and/or rofecoxib was proved by means of controlled oral provocation test to these drugs. In patients with skin symptoms, provocation was considered positive when urticaria–angioedema appeared within 6 h. In patients with respiratory symptoms, provocation was considered positive when a decrease of forced expiratory volume in 1 s greater than 20% than the patient's basal value measured immediately before starting NSAID administration. Thirty subjects with demonstrated tolerance to NSAIDs were selected as controls, after confirmation by means of negative oral provocation. Twenty-one of them were atopic adults and among them 15 were extrinsic asthmatics (Table 2).

Patients with acute, recurrent or chronic urticaria of any other aetiology were excluded. It must be emphasized that none of the patients with NSAID-induced urticaria had a background of urticaria or angioedema of any other kind: they were not ordinary patients with acute or chronic urticaria whose symptoms frequently worsen with NSAIDs. Our respiratory patients were subjects with stable asthma symptoms whose exacerbations were due exclusively to NSAIDs administration. Asthmatic patients with unstable symptoms presenting exacerbations also without NSAID administration were excluded. Subjects with infectious, cardiological, hepatic, and endocrinological diseases were also excluded. Patients stopped the intake of antihistamines at least 5 days before blood sampling (6 weeks for astemizol); none of the asthmatic patients had oral steroid therapy for at least 4 months, but all were under inhalative steroid treatment at the time of blood sampling.

All subjects included in the study were informed orally about the study and signed the corresponding informed consent. The study was approved by the appropriate Ethics Committee.

Flow-cytometric cellular allergen stimulation test

The technique has been described in detail previously [28]. Briefly, after blood cell separation, 50 µL of the patient's cell suspension were incubated with 50 µL of NSAID drugs such as ASA (lysine acetylate, Andromaco, Madrid, Spain) at final concentrations of 1.25 and 0.3 mg/mL; paracetamol (Proparacetamol chlorhydrate, Upsamedica, Madrid, Spain) at 1.25 and 0.3 mg/mL, metamizol (Europharma, Barcelona, Spain)

Table 1. Basophil activation test (% of activated basophils) in NSAID-intolerant patients: individual results

Basophil activation test (FAST) (% activated basophils)																
No.	Clinical reaction	Age/sex	Basal	ASA (mg/mL)		Paracetamol (mg/mL)		Metamizol (mg/mL)		Diclofenac (mg/mL)		Naproxen (mg/mL)		Culprit Drug	Oral Provocation test: Drug and Dose (mg)	Months elapsed last reaction
				1.25	0.31	1.25	0.31	5	0.625	0.31	0.08	1.25	0.31			
1	ASA triad	56/F	2.5	0.3	0.5	0.4	0.3	0.7	1.6	1.3	0.8			IBU	ASA 400	12, 1(P)
2	Cutaneous	20/F	3	3.1	5	0	0.6	1.1	2.1	4.5	2.5			IBU, DIC		3
3	ASA triad	53/F	3.5	2.1	2	0.3	1.2	0.7	1.1	2.5	1.4			ASA, IBU, META		3
4	Cutaneous	42/M	1.5	2.3	1.5	1.5	1.4	0.3	1.7	3.1	1.4			PIRO, INDO, META		48
5	ASA triad	57/F	2.6	6.4	11.5	3.1	3.5	2.3	5	7.6	5.7			ASA, META, IBU		6
6	Cutaneous	61/M	1.2	2	0.3	3.1	1.8	1.6	1	8.4	2.1			ASA (× 3), META		72
7	ASA triad	27/M	2.1	0.6	0.2	0	0.5	0.3	0.2	0	0			Hospital ICU		60
8	Cutaneous	36/M	1.1	2.6	1.0	0.2	1.7	1.1	0.7	9.5	0.6			ASA, META, IBU		2
9	ASA triad	20/F	14	13.0	15.0	3.5	4.5	2.7	1.7	3.8	16.3			ASA, IBU, META		2
10	Cutaneous	34/F	4.1	31.8	3.3	8.9	8.8	1.7	2.4	3.4	5.8			IBU	ASA 150	1(p)
11	Cutaneous	45/F	1.5	2.6	2.7	3.7	1.9	2.8	1.2	3.0	10.2			ASA, META, PROPI	IBU 400	1 (p)
12	ASA triad	60/M	0.6	5.7	0.6	0.3	0.3	0	5.5	2.2	0			ASA	IBU 400	1 (p)
13	Cutaneous	40/M	3	2.6	2.9	2.2	1.4	0.2	0.2	4.3	2.1			ASA, IBU, META		12
14	Cutaneous	32/M	3	1.9	1.6	0	0.9	0.7	0.2	4.4	5.5			ASA (×8)		1 (p)
15	Cutaneous	50/F	3.7	3.9	4.0	2.4	11.1	3.7	2.1	1.7	4.8			ASA (×3), IBU, META		12
16	Asthma	27/M	2	23.1	8.3	1.0	1.9	1.2	4.1	46.3	19.5			ASA (×3) META IBU		36
17	Cutaneous	31/M	7.5	1.1	3.2	0	3.2	1.0	0.42	2.2	2.9			ASA, META, IBU		12
18	ASA triad	35/M	1.1	2.7	1.4	0	0.5	0.1	0.3	2.7	2.8			ASA, META, IBU		22
19	Asthma	38/M	2.4	5.7	4	1.1	1.4	1.8	0.9	41.0	5.7	50.2	8.1	ASA, META, IBU		6
20	Cutaneous	28/M	0.6	53.2	0.6	0.4	2.6	1.1	1.4	58.8	3.1	36.9	0.8	ASA, META, IBU		4
21	Cutaneous	30/F	4.3	33.8	6.0	0.9	0	1.2	0.9	6.1	1.1	3.5	2.6	ASA (×4, IBU)		30
22	Cutaneous asthma	20/F	3.4	19.7	12.3	1.4	1	1.1	0.9	22.5	2.4	24.5	2.1	META (×2), ASA		3
23	Cutaneous asthma	64/F	2.7	0.8	0.8	0	0	0	0.6	2.0	0	10.7	2.2	ASA, IBU, META		12
24	Cutaneous	23/F	0.8	0.2	0.7	0	0	0.3	0.5	1.6	0	0	0.5	META, IBU, ASA, MEFEN	ASA 400	24,
25	Cutaneous	28/F	3.5	5.1	1.8	0	11.8	0	0.5	5.0	0.8	4.3	2.2	DIC	ASA 500	48
26	Cutaneous	30/F	1.8	3.0	1.8	0	0.2	0.2	0	0.9	0	5.4	1.3	ASA, IBU		6
27	Cutaneous	47/F	1.2	9.7	4	0	0.4	0.7	1.2	8.8	1.5	11.1	2.0	ASA (×3), IBU		24
28	Asthma	57/F	0.8	5.1	0.9	0.6	0.3	0	2.2	15.6	1.1	10.8	1.3	ASA (×3), META		36
29	Asthma	52/F	1.6	0.7	0.3	0.3	0.3	1.1	0.5	1.3	0.5	1.0	0.5	ASA, TORADOL		7 yrs
30	Cutaneous	47/F	1.6	8.5	7.1	2.3	1.6	2.5	1.1	11.1	22.7	51.6	5.5	ASA, DIC, IBU		6
31	ASA triad	52/M	10.0	28.5	38.1	2.5	7.4	3.4	10.7	5.4	7.4	12.7	30.4	META, DIC, ASA		28
32	ASA triad	50/F	2.8	12.6	6.5	0.6	0	0	3.1	20.5	3.4	11.8	1.2	ASA, PIRO, META, PARA	ASA 400	1 (p)
33	Cutaneous	56/F	5.1	6.3	8.1	1.3	2.1	0.4	1.9	2.1	1.3	1.9	2.7	ASA, META, PIRO, DIC		22
34	Asthma	58/M	2.3	55.1	41.8	30.8	36.2	10.7	21.1	22.8	34.5	31.2	41.7	META	ASA 400	1 (p)
35	Asthma	64/F	1.2	5.2	2.0	1.3	1.1	1.2	2.7	46.9	5.8	22.9	2.6	META	ASA 150	1 (p)
36	Cutaneous	17/M	5	3.3	10.3	1.4	2.0	3.9	4.1	5.8	3.2	2.4	2.4	ASA	IBU 150	12
37	Cutaneous	45/M	12.5	28	34	32	18	27.1	38.5	33.4	33.5	24.6	29.7	ASA, META, DIC	ASA 400	60

38	Cutaneous	66/F	1.5	4	4.7	1.4	3.0	1.7	4.8	8.8	2.3	3.5	2.3	NAPROX	ASA 500 mg	1(p)
39	Asthma	47/F	1.9	4.3	3.2	0.5	3.7	7.0	6.7	13.6	5.9	7.8	3.2	ASA, META, DIC, others		>12
40	Cutaneous	42/F	2.3	3.8	2.2	1.0	0.7	6.2	7.0	10.7	4.8	5.3	2.5	ASA, META, others		30
41	Cutaneous	22/M	4.1	8.9	7.5	4.1	6.8	13.6	24.4	18.5	16.5	16.9	9.4	ASA (×3), IBU		5 years
42	Cutaneous	65/F	3.8	5.1	4.3	3.3	4.6	2.3	4.0	6.1	4.4	3.7	3.7	DIC, IBU, NAPROX		36
43	Cutaneous	57/M	4.6	9.2	7.3	2.3	4.4	3.2	2.3	10.7	9.0	9.8	11.6	ASA, META, DIC		10 years
44	Cutaneous	65/F	20.3	37.6	42.3	35.1	30	31.4	39.8	26.5	46.8	51.4	40.2	ASA, META, PIRO		>12
45	Cutaneous	55/M	12.3	16.5	10.6	16.3	12.9	9.3	25.1	10.9	30.0	5.5	7.2	ASA, META, DIC		9
46	Cutaneous	42/M	0.5	0.6	0.7	1.1	0	0	0.3	0.6	0.8	0.3	0.8	ASA, META, PIRO, PARA	NIMESULIDE 100	24
47	Asthma	45/F	4.8	5.7	3.1	1.4	1.2	10.1	4.8	44.7	3.6	5.1	3.4	ASA, IBU		2
48	Cutaneous	68/M	0.6	1.2	1.0	2.3	0.7	0	1.0	0.6	1.4	1.7	0.9	ASA, META, DIC		1
49	Cutaneous	47/M	3.7	9.1	15.9	1.1	3.7	0.4	6.4	19.7	8.1	3.0	3.8	ASA, META, DIC		24
50	Cutaneous	46/F	0.3	0.8	2.6	1.4	0.6	0.8	5.8	0.8	3.1	1.6	1.6	ASA, META, DIC	PARA 500	1 (p)
51	Cutaneous	34/F	0.1	5.1	1.4	0.3	0.4	0	0	1.6	0.8	26.8	2.6	ASA, META, DIC		12
52	Cutaneous	31/F	1.8	16.1	12.3	5.3	9.1	4.2	3.2	15.3	18.3	20.1	9.0	META	ASA 500	1 (p)
53	Cutaneous	45/F	1.9	0.5	0	0	1.4	1	0.3	1.4	5.4	0.2	0.2	PIRO, DIC		>5 years
54	Cutaneous	50/F	0.5	0.6	1.6	0.2	0.2	2.2	1.7	0.8	1.2	13.8	5.5	IBU, META, PIRO, KETO		24
55	ASA triad	26/M	15.0	34.0	28.3	24.6	34.1	3.1	3.5	5.0	6.2	3.1	3.4	ASA (×3), PARA (×3)		12
56	Cutaneous	64/M	1.1	9.4	1.4	0.7	0.7	0.6	1.4	2.3	1.3	20.1	1.9	ASA, META, IBU		>5 years
57	ASA triad	58/M	1.2	0.5	0.3	1.4	0.8	1.7	0.6	0.3	1.1	11.6	2.5	ASA, PIRO		>5 years
58	Cutaneous	57/M	3.4	1.6	2.4	0	0.6	0.5	2.1	2.7	0.5	1.4	0	ASA (×3), PROPI	MELOXICAM 15	1 (p)
59	Asthma	26/F	8.6	12.1	9.9	4.4	4.4	0	4.5	0	4.6	1.5	2.1	IBU, ASA		12
60	Cutaneous	58/M	7.2	15.7	6.1	3.8	6.5	2.5	12.2	2.9	4.3	1.9	2.0	IBU, META, DIC	ASA 400	1 (p)

Drugs: ASA, acetylsalicylic acid; DIC, diclofenac; IBU, ibuprofen; META, metamizol; PIRO, piroxicam; PROPI, propifenazone; IND, indomethacin; PARA, paracetamol; MEFEN, metenamic acid; NAPROX, naproxen; KETO, ketoprofen; FAST, flow-cytometric cellular allergen stimulation test; M, male; F, female.

Positive FAST: basophil activation > 5% and Stimulation index > 2.
1(p), positive provocation within 1 month of blood samplings.

Table 2. Basophil activation test (% of activated basophils) in ASA tolerant controls: individual results

No.	Age/sex	Basophil activation test (FAST) (% activated basophils)										
		Basal	ASA (mg/mL)		Paracetamol (mg/mL)		Metamizol (mg/mL)		Diclofenac (mg/mL)		Naproxen (mg/mL)	
			1.25	0.31	1.25	0.31	5	0.625	0.31	0.08	1.25	0.31
1	47/M	0.7	1.5	0.1	0.1	0.2	0.7	0.2	0.8	0.4		
2	16/F	14.4	19.0	19.0	8.2	14.0	15.0	17.3	15.0	14.6		
3	25/H	2.3	2.5	2.8	3.6	4.2	3.8	3.4	2.3	3.2		
4	25/F	11.8	19.5	14.5	2.3	3.6	5.1	3.3	1.5	6.0	1.2	5.3
5	26/F	3.1	1.4	1.7	0.4	0.1	0.3	1.5	1.7	1	2.7	1.6
6	22/F	5.6	2.6	1.7	0	3.0	0	0.8	2.3	0.3	15.1	8.6
7	22/F	0.1	0	0.9	0	0	0	0	0	0.8	4.2	6.2
8	64/M	2.1	2.4	2.8	0.1	0.3	0.2	0.2	0	0.1	1.4	7.2
9	29/F	12.7	3.2	9.2	1.1	6.8	0.9	0.6	0.6	0	0	0.6
10	27/F	0.1	0	0.2	0	0	0	0.2	0	0	4.4	5.8
11	38/F	0.8	0.8	0.6	0.2	0.4	1.2	0.4	1.6	1.5	8.2	1.2
12	41/F	0.1	2.7	1.3	0.8	0	0	0	0.2	1.7	0.2	2.3
13	35/M	2.8	2.4	3.7	0.4	0.6	0.4	0.6	0.4	0.2	0.4	1.6
14	37/M	6.4	5.8	5.4	6.9	1.3	5.6	6.4	8	3.2	2.8	2.5
15	29/F	6.2	9.4	8.9	1.4	8.5	1.5	9.0	8.8	4.8	0.9	0.9
16	18/M	4.3	6.2	4	6.2	2.5	2.5	5	13.4	18.2	3.3	2.4
17	18/M	1.4	0.7	3.4	0	0.7	0	0.9	0.6	0.7	2.6	0.6
18	33/F	2.6	0.7	3.4	0	0.4	0.7	1.7	0	2.3	0.8	0.3
19	59/F	3.2	0.9	1.3	0.9	1.8	0	1.3	1.1	5.0	2.8	3.0
20	41/F	2.3	4.8	4.7	1.0	1.9	1.4	2.9	2.4	4.4	3.1	3.4
21	18/F	2.7	2.4	2.4	0.5	0.6	0	0	53.9	0.6	0.2	22.7
22	26/F	0.4	0.4	1.4	0	1.4	0	0.8	0.6	2.8	0.7	0.3
23	18/F	1.2	2.5	1.6	0.8	0.4	2.9	0.6	2.1	0.7	0.8	0
24	21/F	0.6	2.1	0.4	0.2	0	0.4	0.4	0.3	0	0.3	0.6
25	30/M	2.7	4.7	3.3	0.5	3.4	3.9	1.3	2.6	4.9	6.1	4.7
26	18/F	2.6	1.5	1.1	0	1.3	2.3	3.3	0.8	0	5.4	2.9
27	36/F	3.8	3.8	2.5	0	0.4	0	0	0.9	3.2	4.0	4.7
28	47/F	6.1	6.6	5.9	2.8	4.5	2.1	3.8	8.6	9.3	1.1	4.7
29	27/F	0.1	1.1	1.1	0.3	0.2	0	0.3	1.7	0.2	0.7	0.3
30	18/F	0.1	1.7	0.7	0	0.6	0	0.4	1.3	1.1	1.1	1.2

Positive FAST: activation basophils > 5% and stimulation index > 2.

FAST; flow-cytometric cellular allergen stimulation test; ASA, acetylsalicylic acid.

at 5 and 1 mg/mL, diclofenac (Novartis Farmaceutica SA, Barcelona, Spain) at 0.3 and 0.08 mg/mL and naproxen (Roigfarma, Barcelona, Spain) at 1.25 and 0.3 mg/mL in corresponding wells of a microtitre plate. For ASA and paracetamol, powders were weighed in and solutions prepared daily before use; for the others, commercial injectable solutions were used. These two concentrations were selected as the two highest doses suitable for flow-cytometric cellular allergen stimulation test (FAST) evaluation after performance of more extended dose-response curves in all patients and controls [27].

The NSAIDs tested in the study were selected because of two reasons: firstly because of the high frequency of their prescription in our environment, as in Spain the most used NSAIDs are the salicylates, propionic and aryl-acetic acids, and secondly because we wanted to select a weak NSAID inhibitor of COX such as paracetamol, together with a potent inhibitor such as diclofenac. Unfortunately, we could not include COX-2-selective inhibitors in this study, as they were not available in parenteral formulation for *in vitro* use at the time.

In order to evaluate background basal values without stimulation (negative control), we added merely 50 µL of

stimulation buffer (*N*-2 hydroxyethypeperazine-*N*-2-ethan-sulphonic-acid (HEPES) 20 mM, NaCl 133 mM, KCl 5 mM, CaCl₂ 7 mM, MgCl₂ 3.5 mM, bovine serum albumin 1 mg/mL, pH 7.4), containing IL₃ (2 ng/mL) and heparin (10 µL) (5000 UI/mL; ROVI, Madrid, Spain) to the cell suspension in another well. As positive control, we used a monoclonal anti-IgE antireceptor antibody (Bühlmann, Allschwil, Switzerland) at a final concentration of 1 µg/mL.

The microtitre plate was then incubated at 37 °C for 40 min. The reaction was stopped by adding 100 µL stopping buffer [stopping buffer: HEPES buffer pH 7.3 without calcium or magnesium but containing ethylenediaminetetraacetic acid (HEPES, 20 mM; NaCl, 133 mM; KCl, 5 mM; EDTA, 0.27 mM)] and plates were centrifuged for 5 min at 4 °C, 1000 mg. The basophils from the pellet were then double labelled by adding anti-CD63 phycoerythrin (PE)- and anti-IgE-fluorescein isothiocyanate (FITC) labelled antibodies. After incubation for 30 min at 4 °C, erythrocytes were lysed by incubation for 3 min with Pelilysse Buffer A1 (Johnson & Johnson, Madrid, Spain). Flow cytometric analysis of surface markers was performed at 488 nm on a FACS can flow cytometer equipped with a 15 nW argon ion laser (Becton

Dickinson, Madrid, Spain) and analysed by CellQuest software. On the histogram defined by forward scatter and side scatter, the initial cell gate was defined by a bit map around lymphocytes. The second gate was defined around cells showing high density anti-IgE-label, identifying them as basophils. In each assay, we assessed at least 500 basophils. The other parameter analysed on the identified basophils was CD63. The cut-off points for anti-IgE FITC and anti-CD63 PE were established individually for each patient, according to the initiation of the fluorescence peak in the positive control, threshold values that are usually situated over a fluorescence intensity of $>10^2$.

The reproducibility of the technique was evaluated for 100 determinations performed in 10 patients in duplicate.

Statistical analysis

The data were analysed through the statistical program SPSS 10.0. The comparison of qualitative variables was done by means of the χ^2 test. Reproducibility of FAST results was assessed by the intra-class correlation coefficient. This test measures the reproducibility between two diagnostic techniques with continuous results by creating two new variables: the difference between the determinations and the median of both of them. Results are considered acceptable when this coefficient shows values between 0.4 and 0.75, and excellent when the values are over 0.75. Values $P < 0.05$ were considered statistically significant.

To obtain a specific cut-off point for the significant percentage of activated basophils, we used a receiver operating characteristic (ROC) curve. The optimal cut-off point calculated by ROC curves combines sensitivity and specificity and is the one that shows as co-ordinates the furthest point of the curve from the diagonal. From this, we determined the cut-off point selecting preferentially the optimal specificity values over sensitivity because ASA/NSAID hypersensitivity is a low prevalence pathology. On that basis, results indicating a percentage of basophil activation superior to 5% with a stimulation index (SI: test value/background value) higher than 2 were considered positive for FAST.

The inner validity of the test was assessed by sensitivity (number of patients with positive results/total number of patients), specificity (number of controls with negative results/total number of controls), and positive and negative predictive values.

Results

The reproducibility of the FAST test was evaluated in ten patients, in whom all determinations were performed in duplicate. The intra-class correlation coefficient was excellent, with a value of 0.92 ($P < 0.001$).

All results obtained with five NSAIDs, each at two concentrations, are presented in Table 1 for the 60 patients and in Table 2 for the controls. The ASA, paracetamol, and metamizol used yielded no positive reactions at all in 30 controls, indicating a specificity of 100%. With diclofenac, two controls turned out positive, yielding a specificity of 93.3%.

All the patients were tested with ASA, paracetamol, metamizol, and diclofenac, whose results are shown in Tables 1 and 2. The response to ASA and diclofenac gave the highest sensitivity values (43.3%) with a specificity of 100% and 93.3%, respectively. If we consider these four NSAIDs globally, this technique allows identification of 63.3% of the patients (38/60) with a specificity of 93.3%. The global positive predictive value is 8.7%¹ and the negative predictive value 99.6% (both predictive values are considered over an estimated disease prevalence of 1% in the general population) (Table 3).

Another way to assess the results is to evaluate the pattern of reactivity of the patients and of the controls (one or more positive tests at two concentrations). Among 38 FAST positive patients, 25 (65.8%) were positive to two or more of the possible eight tests (Table 1) while in the controls, only one of the 30 patients was positive to two concentrations of the same NSAID (Table 4). Among 25 patients where the test was performed at less than 12 months after the last known exposure and reaction, the test was positive in 18 (72%). In 35 patients tested 12 months or more after the last known reaction, only 20 (57.1%) were positive ($\chi^2 = 1.39$; $P = 0.24$ n.s.). Eighteen patients underwent positive provocation (Table 1); among the 13 patients having experienced a positive provocation challenge 1 month previous to blood sampling, 10 (76.9%) had a positive FAST test.

If we group the patients according to their cutaneous or airways symptoms, we observe no significant difference when considering the different NSAID globally: for cutaneous symptoms 26 positive/40 (sensitivity 65%), for respiratory symptoms 12/20 (sensitivity 60%). Similar results are obtained when considering each NSAID separately (Table 5).

Forty-two of our patients were tested with naproxen (cases 19–60 from Table 1). The case of naproxen appears to be a special one, because preliminary experiments showed that a higher naproxen concentration induces basophil activation not only in hypersensitive patients but also in a large proportion of the controls (results not shown). At the two naproxen concentrations used, the positive results in patients raise sensitivity to 54.8% but decrease specificity to 74.1%. Adding the two naproxen concentrations in the diagnostic evaluation confirms positive reactions in 19 out of 30 in cases positive to the one or the other NSAIDs (63.3%). In four additional cases (Table 1, # 23, 26, 54, 57) only naproxen is positive, which would bring the global sensitivity to 81% (34/42). This, however, would be accompanied by a global decrease in specificity to 70.4% if all five NSAIDs were included in the evaluation (eight positive/27 controls).

¹The positive predictive value, in low prevalence pathologies, suffers important variations with small modifications in the specificity values, as can be observed, for example, in the results of predictive value of ASA and diclofenac, which have similar values of sensitivity. In the clinical practice of the specialists to whom patients are referred for suspicion of hypersensitivity to NSAIDs and testing, the prevalence of patients with real hypersensitivity to NSAID, like in the current group of patients, will be considerably higher than 1%. Accordingly, in a population with high suspicion of NSAID hypersensitivity, assuming 40% to be really hypersensitive (while in our group it is 100%), the positive predictive value (PPV) for diclofenac would be 90.3% and the PPV for diclofenac would continue to be 100%.

Table 3. Basophil activation test (FAST): sensitivity, specificity, predictive values and likelihood ratios in the diagnosis of NSAID hypersensitivity

	SE	SP	PPV	NPV
Aspirin	26/60 43.3%	30/30 100%	100	99.4
Paracetamol	7/60 11.7%	30/30 100%	100	99.2
Metamizol	9/60 15.0%	30/30 100%	100	99.2
Diclofenac	26/60 43.3%	28/30 93.3%	6.1*	99.4
Naproxen	23/42 54.8%	19/27 74.1%	2.1*	99.4
Aspirin+paracetamol +metamizol+diclofenac	38/60 63.3%	28/30 93.30%	8.7*	99.6

*The low PPV is due to calculation on 1% prevalence in general population; it would be more logical to consider a much higher prevalence in the population tested (see footnote 1).

SE, sensitivity; SP, specificity; PPV, positive predictive value; NPV, negative predictive value; FAST, flow-cytometric cellular allergen stimulation test; NSAID, non-steroidal anti-inflammatory drug.

Table 4. FAST-pattern of reactivity to various NSAIDs in hypersensitivity patients and controls

Number of positive tests*	Patients (n = 60)	Controls (n = 30)
1	13	1
2	10	1
3	6	
4	5	
5	1	
6	1	
7	1	
8	1	

*To four NSAIDs, two concentrations each.

FAST, flow-cytometric cellular allergen stimulation test; NSAID, non-steroidal anti-inflammatory drug.

Table 5. Evaluation of FAST results to NSAIDs according to cutaneous or airways symptoms

	Cutaneous*	Respiratory
Aspirin	16/40 40%	10/20 50%
Paracetamol	5/40 12.5%	2/20 10%
Metamizol	5/40 12.5%	4/20 20%
Diclofenac	17/40 42.5%	9/20 45%
Naproxen	15/30 50%	8/12 66.7%

*Two patients with cutaneous and airways symptoms classified as cutaneous. FAST, flow-cytometric cellular allergen stimulation test; NSAID, non-steroidal anti-inflammatory drug.

Discussion

The diagnostic efficiency of the flowcytometric basophil activation test as described here or by very similar techniques has been well established by clinically validated and controlled studies in recent years with aeroallergens such as pollens and house dust mites [28], food allergens [29, 30], insect venoms [31], and latex [32, 33]. With drugs also, such as betalactams [34, 35], muscle-relaxants [36, 37] and metamizol [38], some studies have been published in the past few years and more extensive evaluation by multi-centric studies is

under way. Some general reviews of the state of the art are available [39, 40].

In the field of hypersensitivity reactions to aspirin and NSAIDs, no controlled and clinically validated study using FAST has yet appeared, despite a number of positive prior anecdotal reports about isolated cases [9, 41]. To our knowledge, this report is the first flowcytometric study encompassing a large series of patients with NSAID hypersensitivity well documented by clinical history presenting at least two clinical episodes because of two different NSAIDs and/or a positive oral challenge. As shown above, the percentage of positive FAST tests varied from one NSAID to the other: 43.3% for ASA and diclofenac, 11.7% for paracetamol, 15% for metamizol, and 52.4% for naproxen when using the two concentrations reported. When considering all first four NSAIDs together, sensitivity increases to 63.3% and to 81% if naproxen is also included. Most importantly, in 30 controls who were to a large majority atopic patients and to 50% asthmatics tolerating NSAIDs, specificity amounted to 100% for ASA, paracetamol and metamizol and, to 93.3% for diclofenac, yielding an overall specificity for the four NSAIDs of 93.3%. Naproxen, on the other hand, is a special case. At higher concentrations than used here, it is an almost obligate basophil stimulant [27] and cannot be used to assess NSAID hypersensitivity. At the two concentrations reported here, however, there is a clear cut difference between patients and controls: 54.8% sensitivity (23 positive/42) vs. 74.1% specificity (20 negative/27) in controls. In practical terms, we would recommend to consider tests with naproxen as optional; they certainly help, when positive with other NSAIDs, to confirm the *in vitro* diagnostic of NSAID hypersensitivity, but, when positive alone, the result should be interpreted with caution. When limiting the tests to the first four NSAIDs, one can take advantage of the high specificity, meaning that any positive test makes the diagnosis of NSAID hypersensitivity quite probable. As sensitivity remains around 65%, a negative test cannot exclude NSAID hypersensitivity, but a positive test is certainly meaningful and in presence of an appropriate clinical history practically confirms the diagnosis. This statement appears the more justified as among the patients tested within 1 month of a positive oral challenge, 76.9% showed a positive FAST test.

It seems important to test not only ASA, but also one or more of the other NSAIDs, because many patients show a pattern of multiple reactivity (two or more positive tests) (Table 4). Indeed, among 38 FAST positive cases to one or several of the first four NSAIDs, 65.8% showed a pattern of multiple reactivity, which is never the case in controls and makes the interpretation of the results much easier. If the number of tests had to be limited, for economic or other considerations, the obvious choice would be ASA and diclofenac, each at the two concentrations indicated, because this combination still yields a sensitivity of 58.3% (35/60), together with a specificity of 93.3%.

It seems optimal to perform the tests within the first 6–12 months following exposure and clinical hypersensitivity reaction to NSAIDs, because in such patients the tests are positive in over 72% of the cases. Patients with a clinical history dating from several years may also be strongly positive but negative tests are more frequently found in that

category (about 42% negative despite very suggestive clinical history). We suggest that these fluctuations are related to the basic cellular and basophil reactivity which accompanies the NSAID syndrome and may be a required basis for its expression [27]. As well documented, basophils from NSAID hypersensitive patients are hyper-reactive non-specifically to C5a and other ligands [24, 42–44]. Clinical hypersensitivity may also fluctuate with time, possibly following the evolution of the underlying inflammation, as shown also by the effect of steroid therapy on the NSAID syndrome. It will be interesting and important to follow patients with basophil reactivity to NSAIDs over a period of years and to assess how far basophil reactivity *in vitro* may correlate with persistence of clinical hypersensitivity to NSAIDs. It would be important to assess whether there is a parallelism in these patients selected for multiple hypersensitivities to NSAIDs, whether there is some parallelism between the reactivity *in vitro* and the reactivity *in vivo*. This point, however, is difficult to investigate systematically, both from ethical and practical point of view because in this study it would have obliged to perform provocation tests to four or five different NSAIDs in each patient. Among 11 patients with positive provocation to ASA, seven showed a positive FAST test (Table 1). From three patients with history of reaction to paracetamol, one showed a positive FAST test to paracetamol. Clarification about the predictive value of a positive or negative FAST test on clinical challenge will require additional quantitative provocation studies.

The precise mechanism(s) of clinical hypersensitivity to NSAIDs and in particular the nature of the main effector cells involved is not yet clear. However, it appears that sulphido-leukotrienes, starting with LTC₄, play a major role in the generation of airways symptoms [3]. Several reports suggest increased production and release of sLTs, already at background levels but even more following exposure to NSAIDs in hypersensitive patients [3, 6, 18]. Up to now, however, there was, to our knowledge, no direct evidence suggesting that blood basophils were also participating in the NSAID hypersensitivity syndrome. Indeed, IgE-mediated allergy in which IgE-sensitized local mast cells are primarily involved almost always involve IgE-sensitized blood basophils as well, but in the case of NSAID hypersensitivity, in which inflammatory cell activation and sLT production are not IgE-dependent, the role of basophils is, up to now, not necessarily manifest.

Indirectly, however, the results of the *in vitro* cysteinyl-leukotriene release test assay indicating overproduction of sLTs by blood leucocytes from a number of NSAID hypersensitive patients with respect to healthy controls and/or of NSAID tolerant asthmatics, following stimulation by NSAIDs *in vitro* [24, 25], have been suggestive of basophil involvement. In NSAID hypersensitive patients, basophils appear to be the main producers of sLTs in peripheral blood [42].

The fact that blood basophils of NSAID hypersensitive patients may become activated and produce, under well-defined and appropriate conditions *in vitro*, more sLTs than those of tolerant patients is becoming increasingly substantiated. This conviction is reinforced by the recent report of Kowalski et al. [45] showing a greater production of 15-hydroxyeicosatetraenoic acid by the same leucocytes from ASA hypersensitive patients stimulated *in vitro*. Various

arguments plead for the mechanism of basophil activation by NSAIDs being different from that of stimulation by IgE and anti-IgE [27].

One probable explanation for this increased sLT production is the inhibition of prostaglandin (PG)E₂ production by COX-1 inhibitors, because PGE₂ is well known as a 'brake' for sLT and mediator release from mast cells and basophils [3]. In addition, the inhibiting effect of PGE₂ on aspirin-stimulated basophils *in vitro* has been reported [25] and the reduced production of PGE₂ from leucocytes of NSAID hypersensitive patients confirmed [46, 47]. Removal of the PGE₂ 'brake' by COX-1 inhibitors on mast cells and basophils is probably, therefore, a key event in the induction of clinical hypersensitivity symptoms. It would also be of interest to investigate whether basophils from hypersensitive patients reacting to COX-1 inhibitors react or not *in vitro* to COX-2 inhibitors such as rofecoxib and celecoxib. These drugs were not available in parenteral form at the very start of the present study and will have to be investigated in the future. The almost obligate basophil activation induced by high concentrations of naproxen even in normal leucocytes is probably also because of removal of the PGE₂ brake [27]. However, other additional trigger mechanisms, such as constitutive increased production of sLTs, increased receptor activity for LTC₄ [46] or diminished lipoxin biosynthesis [48–50] may also play some role.

These theoretical considerations explain the basophil activation in patients with NSAID hypersensitivity. This means that flowcytometry is an *in vitro* diagnostic test with very high specificity and a sensitivity of 60–70%, well correlated with positive challenge tests as described here, could be of definite use to the patients. It will not be a risk and inconvenience to confirm the clinical diagnosis and to avoid provocation, in a sizeable number of cases. When negative, however, it does not allow to exclude NSAID hypersensitivity and provocation challenge remains necessary, if clinically indicated.

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References

- Widal MF, Abrani P, Lermoyez J. Anaphylaxie et idiosyncrasie. Presse Méd 1922; 30:189–92. (Reprinted in: Allergy Proc 1993;14:373–6).
- Samter M, Beers RF. Concerning the nature of intolerance to aspirin. J Allergy 1967; 40:281–93.
- Szczeklik A, Stevenson D. Aspirin-induced asthma: advances in pathogenesis, diagnosis and management. J Allergy Clin Immunol 2003; 111:913–21.
- Kasper L, Sladek K, Duplaga M et al. Prevalence of asthma with aspirin hypersensitivity in the adult population of Poland. Allergy 2003; 58:1064–6.
- Faich GA. Adverse drug reaction monitoring. N Engl J Med 1986; 314:1589–92.

- 6 Szczeklik A, Stevenson DD. Aspirin-induced asthma: advances in pathogenesis and management. *J Allergy Clin Immunol* 1999; 104:5–13.
- 7 Carmona MJ, Blanca M, Garcia A et al. Intolerance to piroxicam in patients with adverse reactions to nonsteroidal antiinflammatory drugs. *J Allergy Clin Immunol* 1992; 90:873–9.
- 8 Himly M, Jahn-Schmid E, Pittertschatscher K et al. IgE-mediated immediate-type hypersensitivity to the pyrazolone drug propyphazone. *J Allergy Clin Immunol* 2003; 111:882–8.
- 9 Sainte-Laudy J. Nine cases of suspected IgE-mediated anaphylaxis induced by aspirin. *ACI Int* 2002; 14:220–2.
- 10 Blanca M, Perez E, Garcia JJ et al. Angioedema and IgE antibodies to aspirin: a case report. *Ann Allergy* 1989; 62:295–8.
- 11 Szczeklik A, Gryglewski RJ, Czerniawska-Mysik G. Relationship of inhibition of prostaglandin biosynthesis by analgesics to asthma attacks in aspirin-sensitive patients. *Br Med J* 1975; 1:67–9.
- 12 Szczeklik A. The cyclooxygenase theory of aspirin-induced asthma. *Eur Respir J* 1990; 3:588–93.
- 13 Stevenson DD, Lewis RA. Proposed mechanism of aspirin sensitivity reactions. *J Allergy Clin Immunol* 1977; 80:788–90.
- 14 Szczeklik A, Sanak M. Molecular mechanisms in aspirin-induced asthma. *ACI Int* 2000; 12:171–8.
- 15 Fischer AR, Rosenberg MA, Lilly CM et al. Direct evidence for a role of the mast cell in the nasal response to aspirin in aspirin-sensitive asthma. *J Allergy Clin Immunol* 1994; 94:1046–56.
- 16 Nasser SM, Lee TH. Leukotrienes in aspirin-sensitive asthma. In: Szczeklik A, Gryglewski RJ, Vane J, eds. *Eicosanoids, aspirin and asthma*. New York: Marcel Dekker, 1998; 317–36.
- 17 Lee TH, Smith CM, Arm JP, Christie PE. Mediator release in aspirin-induced reactions. *J Allergy Clin Immunol* 1991; 88:827–9.
- 18 Stevenson DD, Simon RA. Sensitivity to aspirin and nonsteroidal anti-inflammatory drugs. In: Middleton E, Reed CE, Ellis EF, Adkinson NF, Yunginger JW, Busse WW, eds. *Allergy: Principles and practice*, 5th Edn. St Louis: Mosby, 1998; 1225–34.
- 19 Czech W, Kapp A, Kownatzki E, Schöpf E. Die Bedeutung der *in vitro*-induzierten Histaminfreisetzung aus isolierten Leukozyten für die Diagnostik pseudo-allergischer Reaktionen auf Azetylsalicylsäure. *Allergologie* 1990; 13:201–4.
- 20 Lebel B, Messaad B, Kvedariene V, Rongier M, Bousquet J, Demoly P. Cysteinyl-leukotriene release test (CAST) in the diagnosis of immediate drug reactions. *Allergy* 2001; 56:688–92.
- 21 De Weck AL. Zellulärer Allergen De-Stimulierungs-Test (CAST). Eine Uebersicht und kritische Auswertung der klinischen Anwendung in der Allergiediagnose. *Allergologie* 1997; 20:487–502.
- 22 Kubota Y, Imayama S, Toshitani A et al. Sulfidoleukotriene release test (CAST) in hypersensitivity to nonsteroidal anti-inflammatory drugs. *Int Arch Allergy Immunol* 1997; 114:361–6.
- 23 Mewes T, Riechelmann H, Klimek L. Increased *in vitro* cysteinyl leukotriene release from blood leukocytes in patients with asthma, nasal polyps and aspirin intolerance. *Allergy* 1996; 51:506–10.
- 24 May A, Weber A, Gall H, Kaufmann R, Zollner TM. Means of increasing sensitivity of an *in vitro* diagnostic test for aspirin intolerance. *Clin Exp Allergy* 1999; 29:1402–11.
- 25 Celik G, Bavbeck S, Misirligil Z, Melli M. Release of cysteinyl leukotrienes with aspirin stimulation and the effect of prostaglandin E2 on this release from peripheral blood leukocytes in aspirin-induced asthmatic patients. *Clin Exp Allergy* 2001; 31:1615–22.
- 26 Pierzchalska M, Mastalerz L, Sanak M, Zalula M, Szczeklik A. A moderate and unspecific release of cysteinyl leukotrienes by aspirin from peripheral blood leukocytes precludes its value for aspirin sensitivity testing in asthma. *Clin Exp Allergy* 2000; 30:1785–91.
- 27 Sanz ML, Gamboa PM, de Weck AL. A new combined test with flowcytometric basophil activation (FAST) and determination of sulfidoleukotrienes (CAST) is useful for *in vitro* diagnosis of hypersensitivity to aspirin and other non steroidal anti-inflammatory drugs (NSAIDs). *Int Archs Allergy Clin Immunol* 2004 (in press).
- 28 Sanz ML, Sánchez G, Gamboa PM et al. Ag-induced basophil activation: CD 63 cell expression detected by flow cytometry in allergic patients to *Dermatophagoides pteronyssinus* and *Lolium perenne*. *Clin Exp Allergy* 2001; 31:1007–13.
- 29 Garcia-Aviles C, Sanchez-Lopez G, Sanz ML et al. Flow cytometric Allergen Stimulation Test (FAST) in the *in vitro* diagnosis of food allergy. *Allergy* 2000; 55 (Suppl. 63):128.
- 30 Monneret-Vautrin D, Sainte-Laudy J, Kanny G, Frémont S. Human basophil activation measured by CD63 expression and LTC4 release in IgE-mediated food allergy. *Ann Allergy Asthma Immunol* 1999; 82:33–40.
- 31 Sainte-Laudy J, Sabbah A, Drouet M, Lauret MG, Loiry M. Diagnosis of venom allergy by flow cytometry. Correlation with clinical history, skin tests, specific IgE, histamine and leukotriene C4 release. *Clin Exp Allergy* 2000; 30:1166–71.
- 32 Sanz ML, Gamboa PM, Garcia-Aviles C et al. Flow-cytometric cellular allergen stimulation test in latex allergy. *Int Arch Allergy Immunol* 2003; 130:33–9.
- 33 Ebo DG, Lechkar B, Schuerwegh AJ, Bridts CH, De Clerk LS, Stevens WJ. Validation of a two-color flow cytometric assay detecting *in vitro* basophil activation for the diagnosis of IgE-mediated natural rubber latex allergy. *Allergy* 2002; 57:706–12.
- 34 Sanz ML, Gamboa PM, Uasuf C et al. Flow-cytometric basophil activation test by detection of CD63 expression in patients with immediate type reactions to Beta-lactam antibiotics. *Clin Exp Allergy* 2002; 32:277–86.
- 35 Sanz ML, Gamboa PM, de Weck AL. Clinical evaluation of *in vitro* diagnostic tests in diagnosis of immediate allergic reactions to β -lactam antibiotics. *ACI Int* 2002; 14:185–93.
- 36 Abuaf N, Rajoely B, Ghazouani E et al. Validation of flow cytometry assay detecting *in vitro* basophil activation for the diagnosis of muscle relaxant allergy. *J Allergy Clin Immunol* 1999; 104:411–8.
- 37 Monneret G, Benoit Y, Debard AL, Gutowski MC, Topenot I, Biennu J. Monitoring of basophil activation using CD63 and CCR3 in allergy to muscle relaxant drugs. *Clin Immunol* 2002; 102:192–9.
- 38 Gamboa PM, Sanz ML, Caballero MR et al. Use of CD63 expression as a marker of *in vitro* basophil activation and leukotriene determination in metamizol allergic patients. *Allergy* 2003; 58:312–7.
- 39 Sanz ML, Maselli JP, Gamboa PM et al. Flow-cytometric basophil activation test. A review. *J Invest Allergol Clin Immunol* 2002; 12:143–54.
- 40 De Weck AL, Sanz ML. Flow cytometric Cellular Allergen Stimulation Test (FAST/Flow CAST): technical and clinical evaluation of a new diagnostic test in allergy and pseudo-allergy. *ACI Int* 2002; 14:204–15.
- 41 Sabbah A, Drouet M, Sainte-Laudy J, Lauret MG, Loiry M. Apport de la cytométrie en flux dans le diagnostic allergologique. *Allergie Immunol* 1997; 29:15–21.
- 42 Abrahamsen O, Haas H, Schreiber J, Schlaak M. Differential mediator release from basophils of allergic and non-allergic asthmatic patients after stimulation with anti-IgE and C5a. *Clin Exp Allergy* 2001; 31:368–78.
- 43 Czech W, Schöpf E, Kapp A. Release of sulfidoleukotrienes *in vitro*: Ist relevance in the diagnosis of pseudoallergy to acetylsalicylic acid. *Inflamm Res* 1995; 44:291–5.
- 44 Wedi B, Kapp A. Aspirin induced adverse skin reactions: new pathophysiological aspects. *Thorax* 2000; 55 (Suppl. 2): S70–1.
- 45 Kowalski ML, Ptasińska A, Bienkiewicz B, Pawliczak R, DuBuske L. Differential effects of aspirin and misoprostol on 15-hydroxyeicosa-tetraenoic acid generation by leukocytes from aspirin-sensitive asthmatic patients. *J Allergy Clin Immunol* 2003; 112:505–12.

- 46 Schäfer D, Schmid M, Gode UC, Baenkler BW. Dynamics of eicosanoids in peripheral blood cells during bronchial provocation in aspirin-intolerant asthmatics. *Eur Respir J* 1999; 13:638–46.
- 47 Hecksteden K, Schäfer D, Stuck BA, Klimek L, Hörmann K. Diagnostik der Analgetika-Intoleranz-Syndroms mittels funktioneller Zelltestung (Analgetika-Intoleranz-Test: AIT). *Allergologie* 2003; 26:263–71.
- 48 Serhan CN, Sheppard KA. Lipoxin formation during human neutrophil-platelet interactions. Evidence for the transformation of leukotriene A4 by platelet 12-lipoxygenase *in vitro*. *Proc Natl Acad Sci USA* 1990; 94:9967–72.
- 49 Sanak M, Levy BD, Clish CB et al. Aspirin-tolerant asthmatics generate more lipoxins than aspirin-intolerant asthmatics. *Eur Respir J* 2000; 16:44–9.
- 50 Sousa AR, Parkih A, Scadding G, Corrigan CJ, Lee TH. Leukotriene receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis. *N Engl J Med* 2002; 347:1493–9.